# Fixation and Cryopreservation of Whole Blood and Isolated Mononuclear Cells: Influence of Different Procedures on Lymphocyte Subset Analysis by Flow Cytometry

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**Background: Immunophenotyping of whole blood (WB) and isolated peripheral blood mononuclear cells (PBMCs) is a common tool used to evaluate immune system changes in clinical studies. The development of methods that would allow preservation of samples for flow cytometric analysis is important for the extension of this technology to field testing in settings where the equipment might be not readily accessible.**

**Methods: Three-color flow cytometric analysis was used to determine percentages of T cells and their subsets ($CD3^+$, $CD4^+$, $CD8^+$), B cells ($CD19^+$), and natural killer cells ($CD16^+/56^+$) in WB and PBMCs using variations of a standard stain/fix WB staining procedure (Optilyse) that included staining following fixation and freezing of fixed samples before or after staining.**

**Results: Comparable lymphocyte subset percentages in WB or PBMCs were observed regardless of Optliyse method used (all $P$s $\geq 0.8$). However, differences in fluorescence intensity for several markers were observed across procedures. Compared with the standard stain/fix procedures, fix/stain decreased the mean fluorescence intensities for CD4, CD8, CD19 and CD16/56 in WB and PBMCs ($P \leq 0.03$ for these markers $P = 0.105$ for CD8 in PBMCs). Further decreases in mean fluorescence intensity were seen with the fix/stain/freeze procedure. The stain/fix/freeze yielded intensities largely comparable to those seen with standard stain/fix procedure ($P \geq 0.13$), suggesting that, when the markers of interest are known at the time of field collection, implementation of this procedure might be desirable. Fix/freeze/stain resulted in diminution of intensity in general, but they tended to be more modest than those seen for fix/stain/freeze and therefore might be applicable to field studies in instances when the specific markers of interest cannot be defined upfront.**

**Conclusions: Freezing of fixed WB and PBMCs before or after cell surface staining is a reliable method for preserving specimens in field sites for later determination of lymphocyte subset percentages, which are commonly assessed in immunodeficient and cancer patients.** Published 2004 Wiley-Liss, Inc.†

**Key terms: flow cytometry; whole blood; peripheral blood mononuclear cells; cryopreservation; fixation**

Flow cytometric enumeration of leukocyte subsets in whole blood and isolated peripheral blood mononuclear cells (PBMCs) is a valuable research and clinical tool, in particular for the diagnosis and monitoring of cellular immunodeficiency diseases, leukemia, and lymphomas (1,2). Monitoring of the percentages of various leukocyte populations can be used to determine response to vaccination, follow disease progression or regression, and assess efficacy of treatment in several diseases. The previous decade has witnessed significant advances in the flow cytometric technique of T-cell subset enumeration (3). In clinical cytometry, whole blood lysis methods have substituted for density gradient separation of PBMCs as the routine preparation technique for immunophenotyping as

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a consequence of the decreased requirements for manipulation and blood volume with the former method (4,5). For an accurate assessment of the levels of each lymphocyte population, it is important to stain and analyze blood samples by flow cytometry as soon as possible after collection (6). Significant alteration of cell surface markers may begin to occur soon after a blood sample is collected if steps are not taken to preserve the specimen. This poses an obvious problem for field studies and clinical trials that occur in areas where access to a flow cytometer is restricted. Several reagents and procedures for stabilization and transportation of blood before staining have been developed (7–11). In addition, validation of procedures for storage of processed samples (fixed and stained immediately after collection) is also important. To validate procedures that would allow preservation of fixed and/or stained samples in remote field sites, we have assessed the feasibility and effect of freezing of fixed cells on the detection of cell surface marker expression of standard leukocyte subset markers in cells stained before or after fixation. Because flow cytometers might be unavailable at field sites and analysis of samples in batches at a single center might help avoid laboratory variability, we investigated whether freezing fixed cells would preserve lymphocyte population levels in whole blood and in isolated leukocytes. We also investigated the effect of freezing of stained, fixed samples or fixed, stained samples on this immunophenotyping panel. A standard immunophenotypic panel for determination of percentages of T-cell subsets, B cells, and natural killer (NK) cells (2) was used to compare various procedures for staining, fixation, and cryopreservation of samples.

We propose a simple and reliable fix-and-freeze method that can be performed before or after staining for a conventional lymphocyte panel. This method permits fixation and storage of whole blood or isolated PBMCs without compromise of the percentage of cells that stain positively for standard lymphocyte subset markers, thus allowing the preservation of whole blood leukocytes and isolated PBMCs for lymphocyte subset analysis in central laboratories at later time points.

## MATERIALS AND METHODS

### Sample Collection and Preparation

Peripheral whole blood was collected from 16 normal healthy donors by using acid citrate dextrose (ACD) as an anticoagulant (Baxter, Deerfield, IL, USA). PBMCs were separated by Ficoll density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). PBMCs were washed twice in Dulbecco's phosphate buffered saline (PBS; Gibco, Grand Island, NY, USA). Whole blood was prewashed in Dulbecco's PBS before staining. For staining, $0.5 \times 10^6$ PBMCs or 100 μl of whole blood was used in each tube. In a subset of individuals (n = 8), blood was collected in three different anticoagulants (ethylene-diaminetetra-acetic acid [EDTA], ACD, and heparin).

Table 1
*Panel of Monoclonal Antibodies Used for Immunophenotypic Analysis*

| Antibody panel (clone)[a] | Cells identified |
|---|---|
| IgG1 FITC × IgG1 PE × CD45 PE-Cy5 (X40, X40, HL30) | Isotype control |
| CD3 FITC × CD4 PE × CD45 PE-Cy5 (SK7, SK3, HL30) | CD4 T cells |
| CD3 FITC × CD8 PE × CD45 PE-Cy5 (SK7, SK1, HL30) | CD8 T cells |
| CD3 FITC × CD19 PE × CD45 PE-Cy5 (SK7, 4G7, HL30) | B cells |
| CD3 FITC × CD16 56 PE × CD45 PE-Cy5 (SK7, B73.1, MY31) | Natural killer cells |

[a]PE-Cy5, phycoerythrine 5 conjugate; FITC, fluorescein isothiocyanate; IgG1, immunoglobulin G1; PE, phycoerythrin.

### Flow Cytometric Analysis

Three-color flow cytometric analysis was performed (Coulter, Miami, FL, USA) with EPICS XL software for a panel consisting of anti-CD3, CD4, CD8, CD19, CD16/56 monoclonal antibodies (Becton Dickinson, Franklin Lakes, NJ, USA), as indicated in Table 1. These markers represent a standard immunophenotypic panel for determination of percentages of T-cell subsets, B cells, and NK cells (2). Lymphocytes were gated based on low side scatter and bright CD45 (Becton Dickinson) staining (3,8). These gating strategies were based on flow cytometric analysis guidelines by the Centers for Disease Control and Prevention (CDC) (12) and the National Institute of Allergy and Infectious Disease, Division of Acquired Immunodeficiency syndrome (3).

Five thousand gated events were acquired for each tube. Results are reported as percentage of cells that stained positively for the antibodies of interest and in some cases as mean fluorescence intensity (MFI) expressed in arbitrary units. In some cases, results are reported as absolute counts, which were calculated by multiplying the percentage of positive cells by the lymphocyte absolute counts obtained in a hematologic analyzer (A$^c$T-diff2, Beckman Coulter) using blood collected into EDTA. Immunophenotypic staining was performed as outlined in the following procedures, represented schematically in Figure 1.

**Procedure A: standard stain/fix.** Whole blood or PBMC (100 μl) was added to polypropylene tubes (Becton Dickinson) that contained the antibody combinations listed in Table 1. Antibodies were added as recommended by the manufacturers. Briefly, samples were incubated with antibodies at room temperature for 15 min in the dark, and Optilyse C (500 μl; Immunotech, Marseille, France) or 2% paraformaldehyde solution (500 μl; Sigma, St. Louis, MO, USA) was added for 10 to 15 min, as recommended by the manufacturers. Cells were washed twice in fluorescence-activated cell sorting (FACS) buffer (0.7% bovine serum albumin and 0.01% sodium azide in PBS) and then analyzed within 24 h in a flow cytometer (XL, Beckman Coulter).

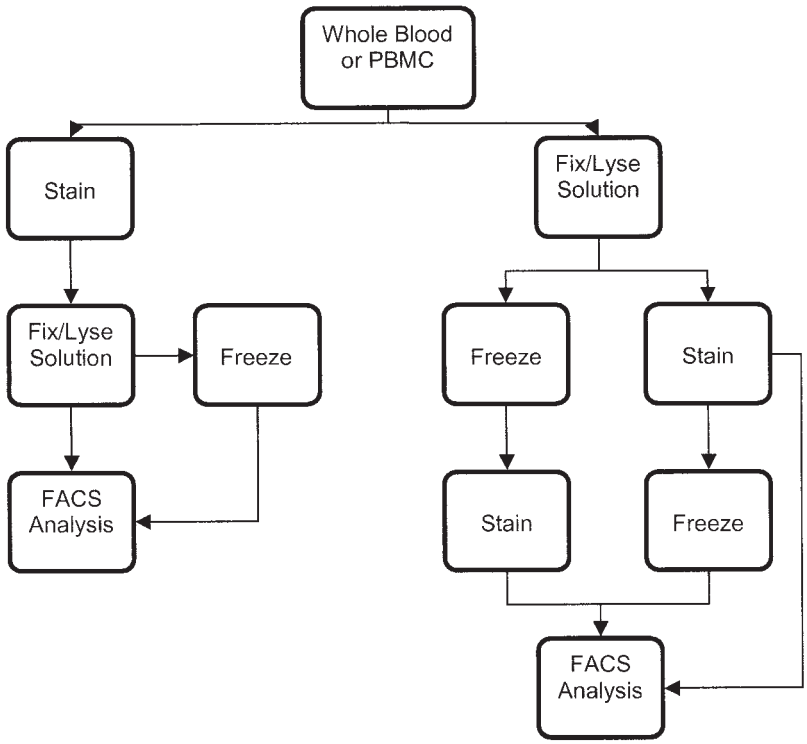


FIG. 1. Diagram with summary of procedures used with whole blood or density gradient isolated PBMCs. See procedures A to E under Materials and Methods.

**Procedure B: fix/stain.** Before addition to tubes containing antibody, whole blood or PBMC was treated as above with Optilyse (15 min in the dark) or 2% paraformaldehyde as a fixative for 10 to 15 min. Samples were then washed twice, stained with the antibodies listed in Table 1, and analyzed within 24 h after staining.

**Procedure C: fix/freeze/stain.** Whole blood or PBMC was fixed for 10 to 15 min and then immediately placed in a liquid nitrogen freezer (−150°C) or −80°C for 1 to 18 days. After thawing rapidly in a water bath at 37°C and two washes with FACS buffer, cells were resuspended in FACS buffer and stained for 15 min in the dark at room temperature with the antibodies listed in Table 1. Storage in a liquid nitrogen freezer or at −80°C produced similar results.

**Procedure D: stain/fix/freeze.** Whole Blood or PBMC was stained with the antibodies listed in Table 1 for 15 min in the dark at room temperature, treated with Optilyse, and then frozen immediately in the fixative in liquid nitrogen (−150°C) or a −80°C freezer. One day and 11 to 18 days later, samples were rapidly thawed in a 37°C water bath, washed twice with FACS wash buffer, resuspended in buffer, and analyzed in the flow cytometer within 24 h as indicated.

**Procedure E: fix/stain/freeze.** Alternatively, whole blood or PBMC was fixed, stained with the antibodies listed in Table 1 for 15 min, washed, resuspended in FACS buffer, and frozen in liquid nitrogen (−150°C) or a −80°C freezer. One day and 11 to 18 days later, cells were rapidly thawed in a water bath at 37°C and read immediately in the flow cytometer.

### Statistical Analysis

Comparisons across treatments were performed with the nonparametric Kruskal-Wallis test. Comparisons between procedures were performed with the Mann-Whitney test. $P < 0.05$ was considered statistically significant.

## RESULTS

In the present study we assessed the extent to which fixation and introduction of a freezing step before or after staining influences the measurement of lymphocyte subsets in whole blood and isolated PBMCs by flow cytometry. By using fixation, freezing, and staining, as diagramed in Figure 1 and described in Materials and Methods, whole blood and PBMC samples from healthy blood donors were evaluated for the lymphocyte panel, which comprised CD3, CD4, CD8, CD19, and CD16/56.

The side scatter characteristics of the lymphocytes (gated based on high CD45 expression and low side scatter) were not markedly affected by freezing or inversion of fixation order in whole blood or PBMC (Fig. 2). However, there was a considerable shift in side scatter for the granulocyte and monocyte populations, which was more marked in whole blood samples (Fig, 2A–E) that were fixed after staining (Fig. 2B) or frozen after fixation (Fig. 2C–E) when compared with the standard method (Fig. 2A), thus producing different profiles for granulocyte and monocyte populations.

Percentages of gated lymphocytes and of cells positive for CD3, CD4, CD8, CD19, and CD16/56 for whole blood and PBMCs treated under the different procedures are

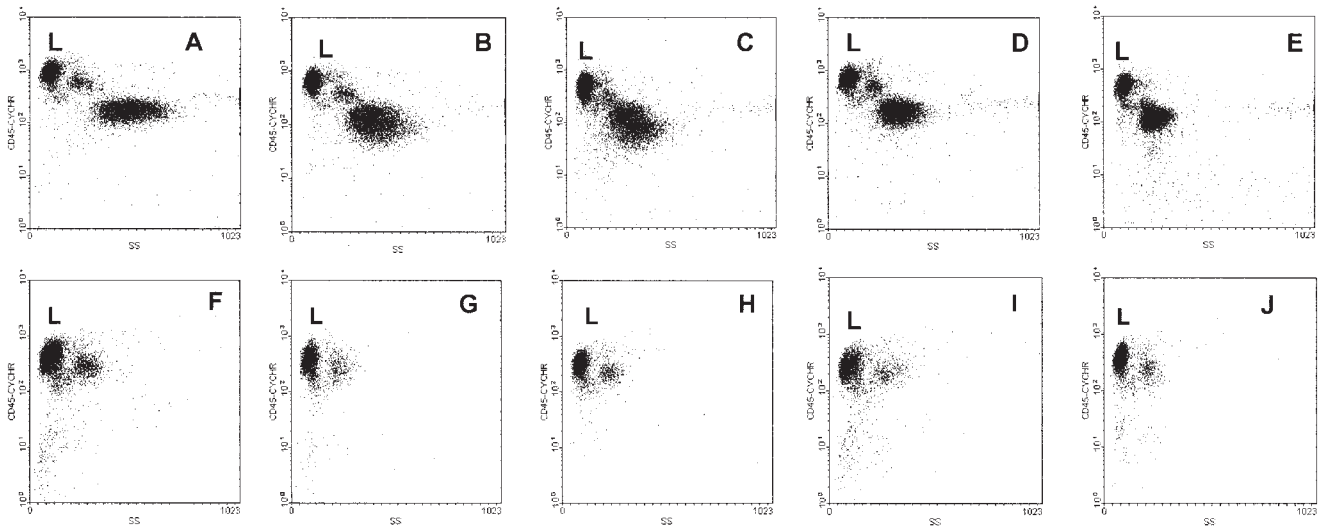


FIG. 2. Influence of the different staining procedures used in side scatter (SS) versus CD45 in whole blood (**A–E**) and PBMCs (**F–J**) treated with Optilyse. Cells were treated with the following procedures: stain/fix (A, F), fix/stain (B, G), fix/freeze/stain (C, H), stain/fix/freeze (D, I), and fix/stain/freeze (E, J). Cells were stained for CD45 in combination with the markers listed in Table 1. Lymphocytes were gated based on bright CD45 and low SS, and this region is indicated by L. Results shown are representative examples from 1 of 16 individuals tested. CD45-CYCHR, CD45 Pycoerythrin-Cyanine 5 conjugate (PE-Cy5).

shown in Figure 3. The percentage of gated lymphocytes was not significantly altered with any of the variant procedures ($P = 0.61$ for whole blood and $P = 0.76$ for PBMC, Kruskal-Wallis test) in whole blood or PBMCs. Lymphocyte subset percentages in whole blood and isolated PBMCs were similar ($P > 0.9$) regardless of whether fixation was performed before or after staining. Lymphocytes that were frozen after fixation and then stained and analyzed produced percentages, for all markers, similar to those exhibited by cells that underwent the standard procedure of stain/fixation for whole blood and PBMC samples ($P \geq 0.8$ for all markers, Mann-Whitney test). Samples treated by stain/fix/freeze yielded similar percentages of lymphocyte markers in whole blood and PBMCs when compared with the standard method of stain/fix ($P \geq 0.8$, Mann-Whitney test; Fig. 3). In addition, fixation of PBMC with 2% paraformaldehyde solution instead of Optilyse did not markedly alter the lymphocyte subset percentages with the different procedures used in two individuals tested ($P > 0.8$, data not shown). Similar results were found when samples were fixed/stained/frozen.

To evaluate whether length of storage in freezer could affect the results obtained with the procedures studied, we tested whole blood and isolated PBMC samples frozen for 11 to 18 days (Fig. 4). No significant differences in the percentage levels of any of the markers tested was observed for storage periods in the freezer up to 18 days ($P \geq 0.42$ for all markers) for whole blood and PBMC. Also, no significant differences in lymphocyte subset percentages were found when samples were frozen in parallel at −80°C or at −150°C in a liquid nitrogen freezer (Fig. 5; $P > 0.57$). This indicates that either freezing condition can be used.

Figure 6 shows the effect of the different procedures of staining on MFI of the different lymphocyte subset markers analyzed in whole blood 1 to 24 h or 11 to 18 days after storage in the freezer. In contrast to percentage values, fluorescence intensity for the different markers

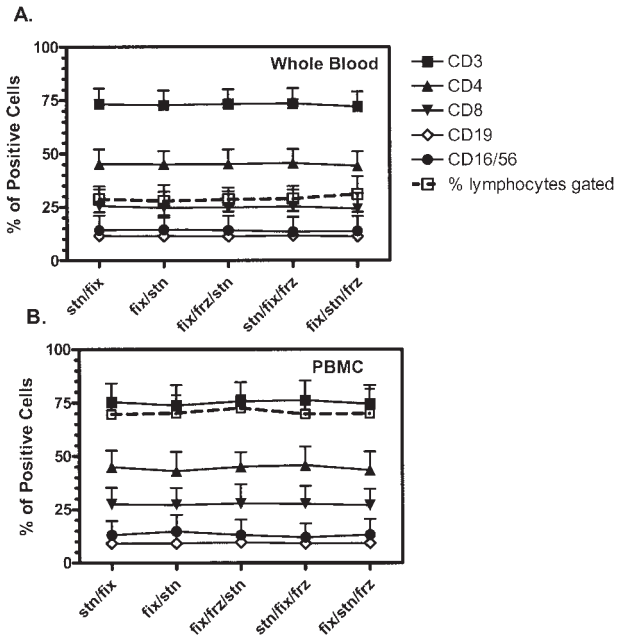


FIG. 3. Influence of different staining procedures on lymphocyte subset percentages (CD3, CD4, CD8, CD19, CD56) gated on bright CD45 and low side scatter (percentage of lymphocytes gated) in whole blood fixed with Optilyse (n = 16 individuals; **A**) and isolated PBMCs fixed with Optilyse (n = 8; **B**). Procedures were performed as described in Materials and Methods. Lymphocyte subset results are expressed as mean percentage of gated cells positive for each marker. Each symbol represents a different marker. Bars above symbols represent standard deviation. frz, freeze; stn, stain.

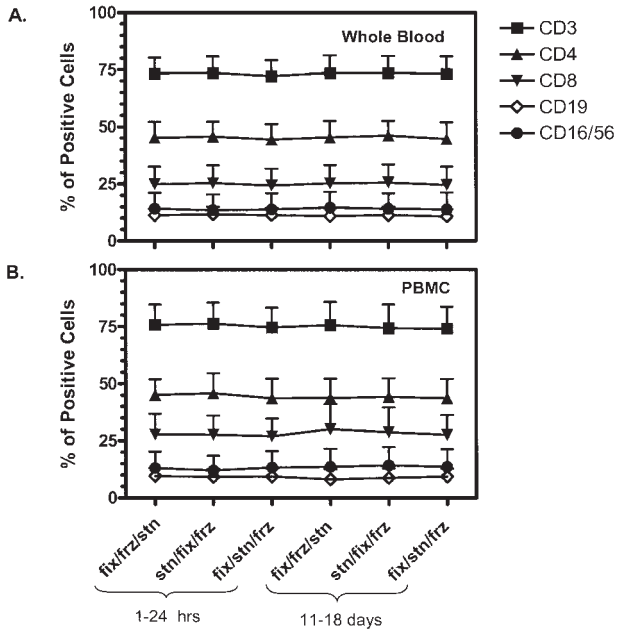


FIG. 4. Influence of time of storage in a freezer for the different procedures in lymphocyte subset percentages in whole blood treated with Optilyse (n = 16 individuals; **A**) and PBMCs treated with Optilyse (n = 8; **B**). Procedures were followed as described in Materials and Methods. Lymphocyte subsets were analyzed in cells that were processed immediately after collection or cells that were stored frozen for 1 to 24 h and for 11 to 18 days. Results are expressed as mean percentage of gated cells positive for each marker. Each symbol represents a different marker. Bars above symbols represent standard deviation. frz, freeze; stn, stain.

analyzed in whole blood (Fig. 6) and in PBMCs (data not shown) varied considerably among the different procedures used ($P < 0.01$ for all, except $P = 0.16$ for CD3 in PBMC and $P = 0.09$ for CD8 in PBMC, Kruskal-Wallis test). The fix/stain method was associated with a decrease in MFI for CD8, CD19, and CD16/56 when compared with the standard stain/fix procedure in whole blood (Fig. 6) and isolated PBMC (data not shown) samples ($P \leq 0.03$ for all markers, except $P = 0.072$ for CD3 in PBMC, $P = 0.105$ for CD8 in PBMC, and $P = 0.08$ for CD3 in whole blood, Mann-Whitney test). The fix/freeze/stain procedure (1 to 24 h of freezer storage) significantly decreased MFI of most markers in whole blood and isolated PBMCs when compared with the standard stain/fix procedure ($P < 0.005$ for all markers except $P = 0.105$ for MFI of CD8 in PBMC and $P = 0.214$ for MFI of CD19 in whole blood). However, this procedure did not affect the MFI as profoundly as the fix/stain/freeze procedure, which consistently resulted in the lowest fluorescence intensity for CD4, CD8, CD19, and CD16/56 in whole blood (Fig. 6) and for CD8, CD19, and CD16/56 in PBMC (data not shown). The stain/fix/freeze (1 to 24 h of freezer storage) procedure did not markedly affect the intensity of fluorescence of any of these markers in whole blood (Fig. 6) or PBMC (data not shown) when compared directly with the standard stain/fix procedure ($P \geq 0.13$ for all markers, Mann-Whitney test). Intensity of fluorescence was not significantly affected by storage length when samples stored frozen for 11 to 18 days were compared with samples frozen for up to 24 h in whole blood (Fig. 6) and in PBMC (data not shown) samples ($P \geq 0.08$, Mann-Whitney test), except for CD16/56 in whole blood for stain/fix/freeze and fix/freeze/stain procedures ($P = 0.04$ and $P = 0.03$, respectively). In addition, there was a tendency for a decrease in average MFI over time, in particular for CD8 in whole blood (Fig. 6).

We then addressed whether these procedures could be applied to blood collected with different anticoagulants (ACD, EDTA, and heparin) that are useful for different research studies. The different procedures of fixation and freezing yielded similar lymphocyte subset percentages for each anticoagulant used ($P > 0.99$; Fig. 7), suggesting that the procedures described in this report can be applied to bloods collected in different anticoagulants.

CDC guidelines recommend collection of blood into EDTA tubes for determination of leukocyte counts by using a hematology analyzer, even when immunophenotyping is done in blood collected in other anticoagulants (12). Immunophenotyping in Optilyse-fixed

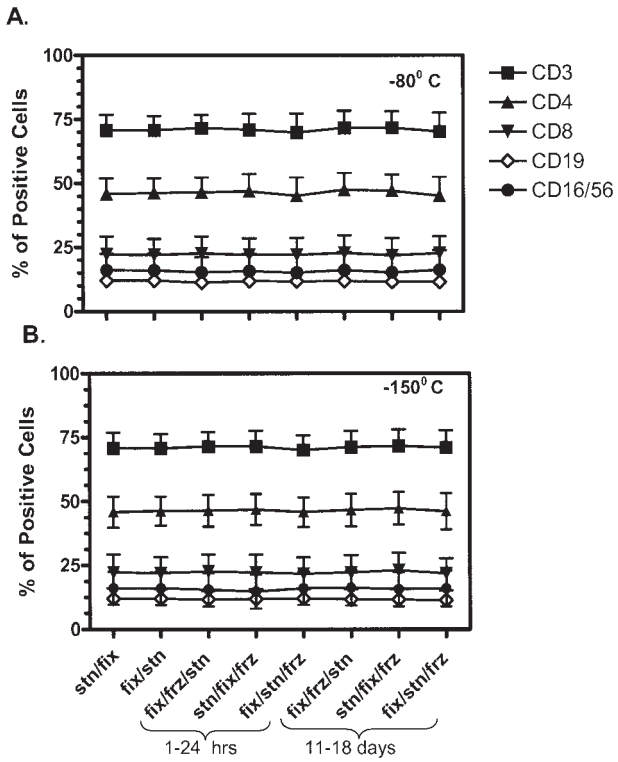


FIG. 5. Influence of different procedures in lymphocyte subset percentages in whole blood treated with Optilyse frozen at −80°C (**A**) or in a liquid nitrogen freezer at −150°C (**B**). Lymphocytes were gated based on bright CD45 and low side scatter. Lymphocyte subsets were analyzed in samples that were processed immediately after collection or cells that were stored frozen for 1 to 24 h and for 11 to 18 days. Results are expressed as mean percentage of gated cells positive for each marker. Each symbol represents a different marker. Bars above symbols represent standard deviation. frz, freeze; stn, stain.

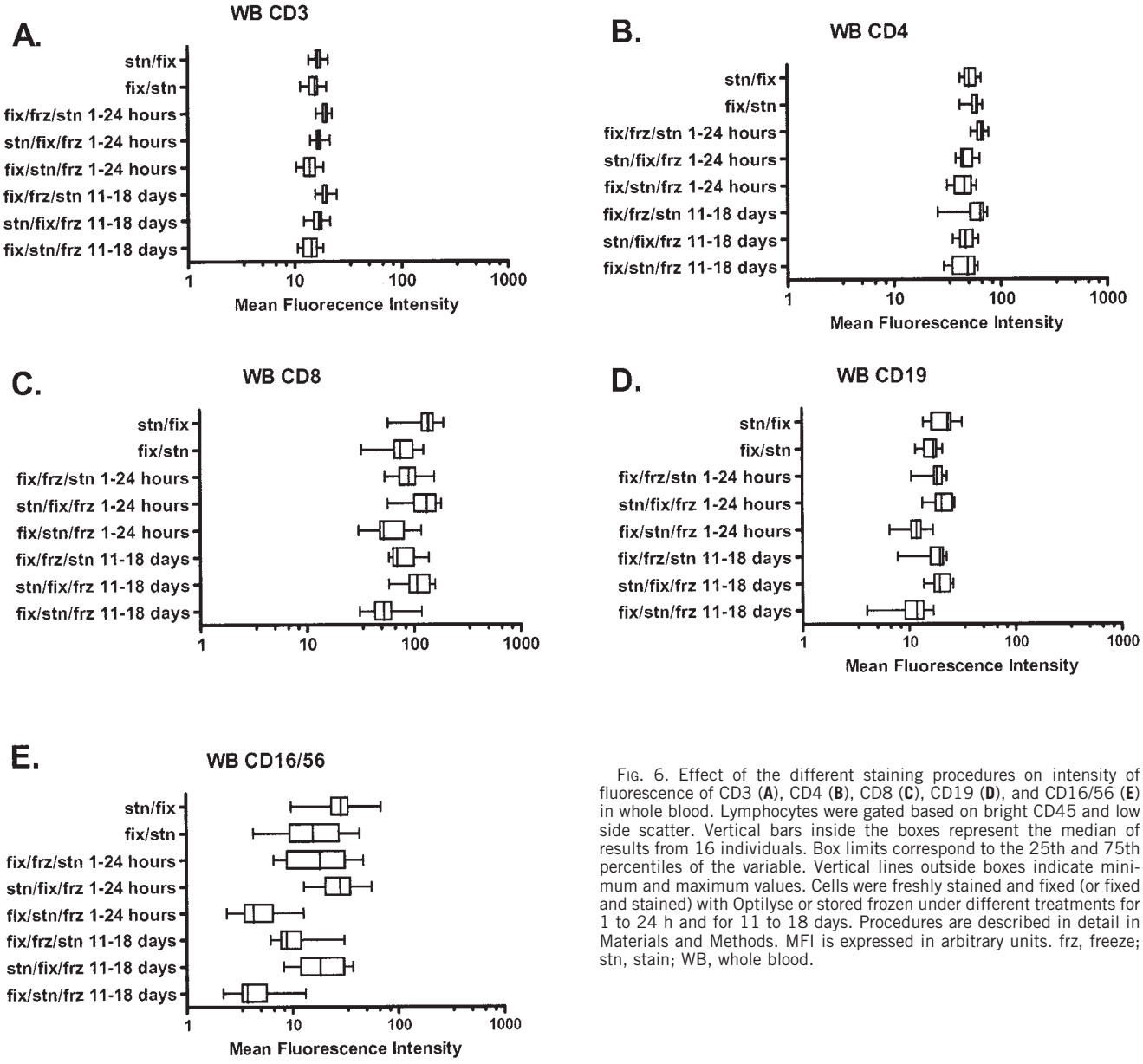


FIG. 6. Effect of the different staining procedures on intensity of fluorescence of CD3 (**A**), CD4 (**B**), CD8 (**C**), CD19 (**D**), and CD16/56 (**E**) in whole blood. Lymphocytes were gated based on bright CD45 and low side scatter. Vertical bars inside the boxes represent the median of results from 16 individuals. Box limits correspond to the 25th and 75th percentiles of the variable. Vertical lines outside boxes indicate minimum and maximum values. Cells were freshly stained and fixed (or fixed and stained) with Optilyse or stored frozen under different treatments for 1 to 24 h and for 11 to 18 days. Procedures are described in detail in Materials and Methods. MFI is expressed in arbitrary units. frz, freeze; stn, stain; WB, whole blood.

bloods requires reference to absolute counts from blood collected in EDTA tubes before any treatment. Fixation with Optilyse involves several steps of dilutions and washes and therefore requires the use leukocyte counts from untreated, undiluted blood. However, to confirm that fixing and freezing would not affect total white blood cell counts, we determined total leukocyte numbers in untreated blood or blood treated with Optilyse before or after freezing. Comparable white blood cell counts were observed ($8.03 \pm 1.85 \times 10^3$, $7.9 \pm 2.16 \times 10^3$, $7.7 \pm 2.02 \times 10^3$ cells/μl for unfixed, Optilyse-fixed, or fixed/frozen blood, respectively). Immunophenotyping results, expressed in absolute cell counts for the three anticoagulants tested, are presented in Table 2.

## DISCUSSION

Several studies have investigated the benefits of different types of reagents (13–15) designed to fix leukocytes and lyse erythrocytes, to enable direct immunophenotyping of whole blood. Standard whole blood immunophenotyping procedures for flow cytometry involve staining with specific monoclonal antibodies, lysis of the erythrocytes, and fixation of leukocytes. Flow cytometric analysis is usually performed within 24 h after the staining procedure. In the present study, the fixatives used were the commercially available Optilyse C for whole blood and isolated PBMCs. Optilyse C procedures use 1.5% formaldehyde and an erythrocyte-lysing agent. In this work, we report and validate variations of the standard Optilyse

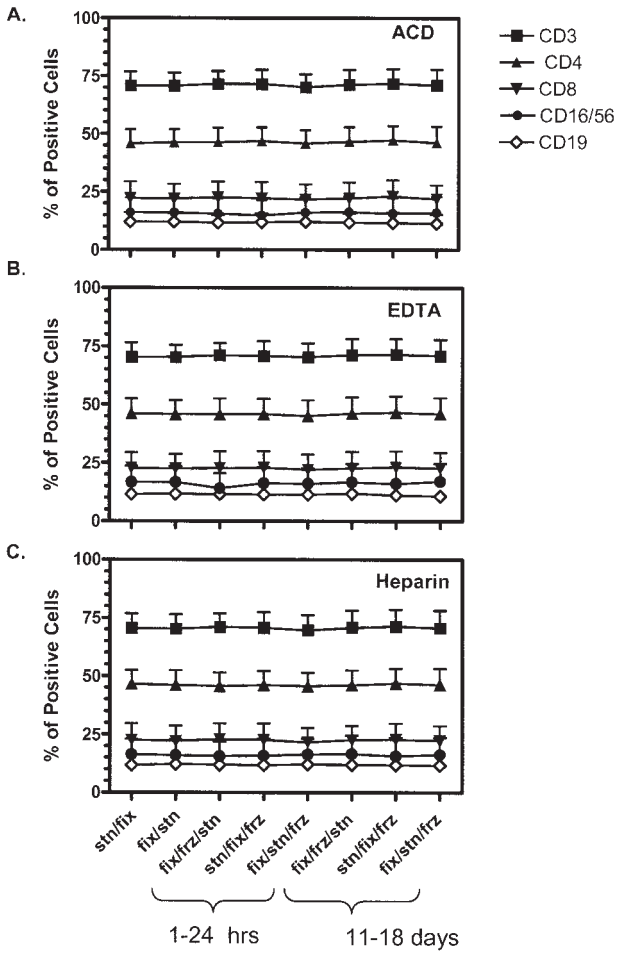


FIG. 7. Effect of different staining procedures on lymphocyte subset percentages on bloods collected using ACD (**A**), EDTA (**B**), or heparin (**C**). Procedures are described in detail in Materials and Methods. Lymphocyte subsets were analyzed in cells that were processed immediately after collection or cells that were stored frozen for 1 to 24 h or for 11 to 18 days. Results are expressed as mean percentage of gated cells positive for each marker. Each symbol represents a different marker. Bars above symbols represent standard deviation. frz, freeze; stn, stain.

method used for whole blood analysis of lymphocyte subset markers that allow transportation of samples from the field to central flow cytometric laboratories or storage until a larger batch is accumulated, without significant alterations in the enumeration of major lymphocyte subsets. However, the wide applicability of these procedures is limited to field sites that are equipped with a freezer and minimal laboratory equipment.

The first question addressed with this study was to determine whether samples could be fixed before staining by using standard methods of whole blood fixation. Our results demonstrated that staining of fixed cells produced results similar to those of staining before fixation with regard to percentages of cells positive for CD3, CD4, CD8, CD19, and CD16/56. These results indicate that it is possible to fix before staining without significant compromise of the relative percentages obtained.

Second, we tested whether fixed whole blood or isolated PBMCs could be frozen in fixative and, hence, stored until staining and flow cytometric analysis. Our results demonstrated that freezing of fixed whole blood or isolated PBMCs did not cause significant changes in the phenotype of peripheral blood lymphocytes but rather produced percentages similar to those obtained with the standard method. Interestingly, we also found that freezing (at −80°C or −150°C) of fixed, previously stained cells is suitable for storage of samples for flow cytometric analysis of lymphocyte subset markers because it did not seem to significantly affect the percentages of expression of any of the markers tested. The overall pattern of linear side scatter versus CD45 found for lymphocytes with the several variant methods resemble the pattern observed with the standard method. However, monocyte and granulocyte profiles were altered by the variant procedures, indicating that the feasibility of application of these procedures to the analysis of leukocyte populations other than lymphocytes needs to be examined. Although the percentages of positive cells for the different markers were not affected by altering the order of fixation or introduction of a freezing step in the protocol, intensity of fluorescence was affected by these variant procedures, indicating that fixation before staining and freezing might adversely affect the intensity of signal obtained. Our results indicated that fixation before staining is associated with a decrease in MFI for CD8, CD19, and CD16/56 when compared with the standard stain/fix procedure. Further decreases on MFI were seen when samples were frozen after fixation and staining (fix/stain/freeze). The fix/stain/freeze procedure was the method that yielded the lowest MFI in whole blood and in PBMC when compared with the standard method of stain/fix or fix/stain. The decrease in MFI associated with fixation before staining may be related to fixative-induced alterations in antibody binding capacity due to potential alterations on epitope exposure and affinity, so it is reasonable to expect that different markers would be differentially affected by the fixative. Consistent with this interpretation, MFI after fix/stain was lower than that after stain/fix for several markers, even in the absence of freezing. Freezing seems to adversely affect MFI for already fixed/stained samples for several markers; however, when staining was performed after freezing, MFI was not compromised when compared with fixed/stained samples. These results suggest that a cycle of freeze/thaw may affect signal intensity of antibodies bound to already fixed cells and thus did not undergo cross-linking by the fixative. In addition, alterations in pH, incubation temperature, lysing reagents, fluorochrome, and antibody titer can have significant effects on results of antibody binding capacity (16). For an accurate evaluation of the effects of procedures on antigen density, quantitative flow cytometry that uses calibration standards will be required for the markers of interest (17).

Third, we addressed whether storage length (up to 18 days) would affect the results produced by each procedure. No significant differences regarding percentage of major lymphocyte subsets were seen in cells processed up

Table 2
*Lymphocyte Subset Absolute Cell Counts for Each Procedure Used in Blood Collected in Three Different Anticoagulants (ACD, EDTA, and Heparin)*

| Treatment | Freeze length | Freeze temperature (°C) | Absolute counts ($\times 10^3$ cells/μl) CD3 | CD4 | CD8 | CD19 | CD16/56 |
|---|---|---|---|---|---|---|---|
| ACD | | | | | | | |
| Stain/fix | Fresh | — | 1.251 ± 0.115 | 0.812 ± 0.236 | 0.391 ± 0.141 | 0.209 ± 0.072 | 0.279 ± 0.115 |
| Fix/stain | Fresh | — | 1.250 ± 0.113 | 0.820 ± 0.234 | 0.386 ± 0.130 | 0.208 ± 0.069 | 0.278 ± 0.113 |
| Fix/freeze/stain | 1–24 h | −80 | 1.263 ± 0.107 | 0.823 ± 0.239 | 0.398 ± 0.144 | 0.197 ± 0.065 | 0.266 ± 0.107 |
| Stain/fix/freeze | 1–24 h | −80 | 1.253 ± 0.117 | 0.830 ± 0.245 | 0.389 ± 0.138 | 0.206 ± 0.076 | 0.272 ± 0.117 |
| Fix/stain/freeze | 1–24 h | −80 | 1.237 ± 0.113 | 0.802 ± 0.255 | 0.387 ± 0.134 | 0.204 ± 0.071 | 0.259 ± 0.113 |
| Fix/freeze/stain | 1–24 h | −150 | 1.264 ± 0.109 | 0.821 ± 0.240 | 0.397 ± 0.141 | 0.203 ± 0.063 | 0.267 ± 0.109 |
| Stain/fix/freeze | 1–24 h | −150 | 1.262 ± 0.122 | 0.829 ± 0.239 | 0.392 ± 0.144 | 0.206 ± 0.071 | 0.259 ± 0.122 |
| Fix/stain/freeze | 1–24 h | −150 | 1.238 ± 0.109 | 0.811 ± 0.225 | 0.382 ± 0.126 | 0.211 ± 0.073 | 0.278 ± 0.109 |
| Fix/freeze/stain | 11–18 d | −80 | 1.267 ± 0.119 | 0.841 ± 0.244 | 0.402 ± 0.138 | 0.208 ± 0.072 | 0.278 ± 0.119 |
| Stain/fix/freeze | 11–18 d | −80 | 1.266 ± 0.135 | 0.833 ± 0.242 | 0.383 ± 0.130 | 0.200 ± 0.077 | 0.267 ± 0.135 |
| Fix/stain/freeze | 11–18 d | −80 | 1.243 ± 0.140 | 0.804 ± 0.257 | 0.400 ± 0.132 | 0.199 ± 0.077 | 0.282 ± 0.140 |
| Fix/freeze/stain | 11–18 d | −150 | 1.257 ± 0.125 | 0.826 ± 0.236 | 0.388 ± 0.137 | 0.205 ± 0.075 | 0.283 ± 0.125 |
| Stain/fix/freeze | 11–18 d | −150 | 1.265 ± 0.124 | 0.835 ± 0.237 | 0.405 ± 0.141 | 0.204 ± 0.080 | 0.274 ± 0.124 |
| Fix/stain/freeze | 11–18 d | −150 | 1.256 ± 0.122 | 0.820 ± 0.252 | 0.382 ± 0.119 | 0.198 ± 0.068 | 0.277 ± 0.122 |
| EDTA | | | | | | | |
| Stain/fix | Fresh | — | 1.239 ± 0.122 | 0.816 ± 0.244 | 0.400 ± 0.140 | 0.202 ± 0.072 | 0.292 ± 0.122 |
| Fix/stain | Fresh | — | 1.240 ± 0.123 | 0.810 ± 0.233 | 0.396 ± 0.135 | 0.205 ± 0.072 | 0.292 ± 0.123 |
| Fix/freeze/stain | 1–24 h | −80 | 1.261 ± 0.104 | 0.800 ± 0.233 | 0.403 ± 0.142 | 0.194 ± 0.069 | 0.274 ± 0.104 |
| Stain/fix/freeze | 1–24 h | −80 | 1.242 ± 0.116 | 0.803 ± 0.227 | 0.400 ± 0.140 | 0.198 ± 0.069 | 0.280 ± 0.116 |
| Fix/stain/freeze | 1–24 h | −80 | 1.224 ± 0.118 | 0.787 ± 0.255 | 0.390 ± 0.128 | 0.201 ± 0.073 | 0.289 ± 0.118 |
| Fix/freeze/stain | 1–24 h | −150 | 1.255 ± 0.080 | 0.809 ± 0.237 | 0.401 ± 0.145 | 0.201 ± 0.068 | 0.234 ± 0.080 |
| Stain/fix/freeze | 1–24 h | −150 | 1.250 ± 0.126 | 0.810 ± 0.236 | 0.403 ± 0.151 | 0.200 ± 0.072 | 0.284 ± 0.126 |
| Fix/stain/freeze | 1–24 h | −150 | 1.241 ± 0.119 | 0.803 ± 0.249 | 0.388 ± 0.128 | 0.200 ± 0.070 | 0.281 ± 0.119 |
| Fix/freeze/stain | 11–18 d | −80 | 1.253 ± 0.128 | 0.816 ± 0.243 | 0.407 ± 0.147 | 0.201 ± 0.073 | 0.288 ± 0.128 |
| Stain/fix/freeze | 11–18 d | −80 | 1.254 ± 0.129 | 0.822 ± 0.246 | 0.386 ± 0.121 | 0.197 ± 0.072 | 0.278 ± 0.129 |
| Fix/stain/freeze | 11–18 d | −80 | 1.241 ± 0.149 | 0.804 ± 0.266 | 0.401 ± 0.130 | 0.188 ± 0.077 | 0.287 ± 0.149 |
| Fix/freeze/stain | 11–18 d | −150 | 1.256 ± 0.130 | 0.819 ± 0.247 | 0.400 ± 0.146 | 0.206 ± 0.074 | 0.290 ± 0.130 |
| Stain/fix/freeze | 11–18 d | −150 | 1.258 ± 0.126 | 0.823 ± 0.247 | 0.403 ± 0.137 | 0.196 ± 0.075 | 0.279 ± 0.126 |
| Fix/stain/freeze | 11–18 d | −150 | 1.251 ± 0.137 | 0.814 ± 0.240 | 0.398 ± 0.132 | 0.187 ± 0.084 | 0.294 ± 0.137 |
| Heparin | | | | | | | |
| Stain/fix | Fresh | — | 1.245 ± 0.111 | 0.826 ± 0.235 | 0.395 ± 0.145 | 0.204 ± 0.070 | 0.284 ± 0.111 |
| Fix/stain | Fresh | — | 1.242 ± 0.110 | 0.817 ± 0.235 | 0.388 ± 0.131 | 0.211 ± 0.069 | 0.276 ± 0.110 |
| Stain/fix/freeze | 1–24 h | −80 | 1.245 ± 0.116 | 0.815 ± 0.246 | 0.388 ± 0.134 | 0.207 ± 0.069 | 0.280 ± 0.116 |
| Fix/stain/freeze | 1–24 h | −80 | 1.229 ± 0.116 | 0.798 ± 0.264 | 0.385 ± 0.124 | 0.219 ± 0.073 | 0.274 ± 0.116 |
| Fix/freeze/stain | 1–24 h | −80 | 1.261 ± 0.096 | 0.814 ± 0.224 | 0.394 ± 0.136 | 0.205 ± 0.061 | 0.259 ± 0.096 |
| Stain/fix/freeze | 1–24 h | −150 | 1.248 ± 0.128 | 0.817 ± 0.238 | 0.397 ± 0.141 | 0.203 ± 0.070 | 0.274 ± 0.128 |
| Fix/stain/freeze | 1–24 h | −150 | 1.231 ± 0.109 | 0.810 ± 0.245 | 0.377 ± 0.127 | 0.209 ± 0.073 | 0.282 ± 0.109 |
| Fix/freeze/stain | 1–24 h | −150 | 1.256 ± 0.110 | 0.807 ± 0.234 | 0.401 ± 0.135 | 0.207 ± 0.074 | 0.269 ± 0.110 |
| Stain/fix/freeze | 11–18 d | −80 | 1.224 ± 0.098 | 0.821 ± 0.245 | 0.383 ± 0.120 | 0.198 ± 0.072 | 0.244 ± 0.098 |
| Fix/stain/freeze | 11–18 d | −80 | 1.249 ± 0.142 | 0.811 ± 0.246 | 0.397 ± 0.124 | 0.202 ± 0.076 | 0.273 ± 0.142 |
| Fix/freeze/stain | 11–18 d | −80 | 1.262 ± 0.132 | 0.827 ± 0.238 | 0.399 ± 0.138 | 0.217 ± 0.076 | 0.279 ± 0.132 |
| Stain/fix/freeze | 11–18 d | −150 | 1.257 ± 0.138 | 0.829 ± 0.243 | 0.400 ± 0.144 | 0.203 ± 0.078 | 0.271 ± 0.138 |
| Fix/stain/freeze | 11–18 d | −150 | 1.249 ± 0.116 | 0.820 ± 0.245 | 0.388 ± 0.125 | 0.201 ± 0.063 | 0.278 ± 0.116 |
| Fix/freeze/stain | 11–18 d | −150 | 1.251 ± 0.133 | 0.817 ± 0.243 | 0.396 ± 0.133 | 0.208 ± 0.076 | 0.284 ± 0.133 |

to 18 days after freezing when compared with cells frozen for 24 h. These results guarantee enough time to allow transportation from a remote site to a central flow laboratory.

Previous reports have identified reagents, such as TransFix or Cyto-Chex, that allow storage (7 of 10 days) of whole blood at room temperature or at 4°C (7–11). These reagents have proven to deliver reliable lymphocyte subset results. In this work, we describe procedures that allow freezing of fixed or previously stained samples by using a standard, commercially available method for phenotypic analysis. We are currently addressing whether longer storage periods in the freezer affect staining results. Therefore, freezing of stained or unstained fixed cells before flow cytometric analysis is feasible without marked changes in the phenotype of the markers analyzed. Interestingly, these procedures can be applied to bloods collected with different anticoagulants (ACD, EDTA, and heparin). It is important to note that the markers used in this study identify distinct bright populations. Therefore, depending on the marker of interest, fix/freeze procedures may or not be appropriate depending on the extent to which they affect expression. We are currently testing whether intensity of expression of additional markers within the lymphocyte subsets (such as activation markers) are affected by the variant methods described here. It is also important to note that, for determination of absolute cell counts, these procedures rely on a hematology analyzer at the field site to determine white blood cell counts on untreated, freshly drawn blood.

In summary, variants of the standard Optilyse procedure can be used for determination of percentages of T, B, and NK cells in blood and isolated PBMCs by flow cytometry. The immunophenotyping procedures described in this report present several advantages in clinical settings. These methods will enable preparation of samples that can be stored fixed and frozen for future staining, thus allowing testing of new markers that may have not preselected at the time of collection. In addition, studies with predetermined panels can be processed by the stain/fix/freeze method at the site of collection and then transported in batches to laboratories where flow cytometers are available for analysis. These findings may therefore have an effect in lymphocyte phenotyping in field studies where flow cytometers might be not accessible. These methods allow flow cytometric analysis to be extended to field testing of lymphocyte markers.

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